

# Reproductive Proteomics Comes of Age

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When it comes to sex, Flaubert's prescient comment, "Le bon Dieu est dans le détail" hits the mark. Although "Diable" is often substituted, this poignant statement of the human condition also applies to the study of sexual reproduction. At all levels of human effort, the devil is indeed in the details, and nowhere are the "details" as convoluted, diversified, and obscure as found in the study of the biology of reproduction. Indeed, from the dawn of modern cell biology and the unfolding of the genetic basis of inheritance, the greatest biologists of the 17th and 18th centuries were almost obsessively focused on the biology of sex (or regeneration as it was known at the time). Summarized magnificently in E.B. Wilson's iconic treatise, "The Cell in Development and Heredity" (1), our obsession with sex stems in part from one undeniable fact—organismal fitness and species survival depend exclusively on the faithful replication and subsequent viability of their offspring. As such, sexual reproduction can be considered a *sine qua non* to biological life and a central force in driving evolutionary processes. As first envisioned in his theory of sexual selection (2), Darwin, and a legion of biologists that followed, initially focused on observable morphologies associated with secondary sexual traits (peacock feathers, antlers, horns of beetles, etc.). However, beginning with Parker's brilliant theory of sperm competition in the 1970s (3), a generation of reproductive and evolutionary biologists were inspired to focus on a specific cell—the spermatozoa (reviewed in (4)). Coincident with these efforts, cell and developmental biologists were busy identifying dozens of specific proteins required for sperm-egg interactions and fertilization (reviewed in (5)). The two disciplines—evolution and cell biology—although representing essentially polar opposite approaches, with the former "top down" and the latter "bottom up," have yielded important breakthroughs in our understanding of sexual reproduction. Therefore, it can be argued that elucidating the "details" of sexual reproduction across the tree of life will provide significant insights not only into the intimate molecular mechanism involved, but also inform us about the very fundamental evolutionary processes from which sex evolved. Although we are very far from an evolutionary "grand synthesis" of sexual reproduction, one goal of this issue of *Molecular and Cellular Proteomics* is to highlight the efficacy of proteomics in elucidating these deeper "details" of sexual reproduction.

Fig. 1 ((6) reprinted with permission) attempts to convey the magnitude and complexities of sexual reproduction, and it served as inspiration for the issue's cover art (see inside cover for a description). Both figures illustrate the complexities of sexual reproduction from the biological and human historical perspectives both inexorably intertwined and inseparable. Fig. 1 also summarizes the enormous focus on fertilization by past generations of cell and developmental biologists that has produced a prodigious body of knowledge of the molecular basis of fertilization. This previous work has paved the way for the high throughput technologies available for functional genomic and systems level analyses available today, including mass spectrometry and proteomics approaches. The contributions in this issue of *Molecular & Cellular Proteomics* are intended to showcase the power of proteomics to discover new pathways and processes, and to tackle existing problems and areas of sexual reproduction previously refractory to conventional approaches.

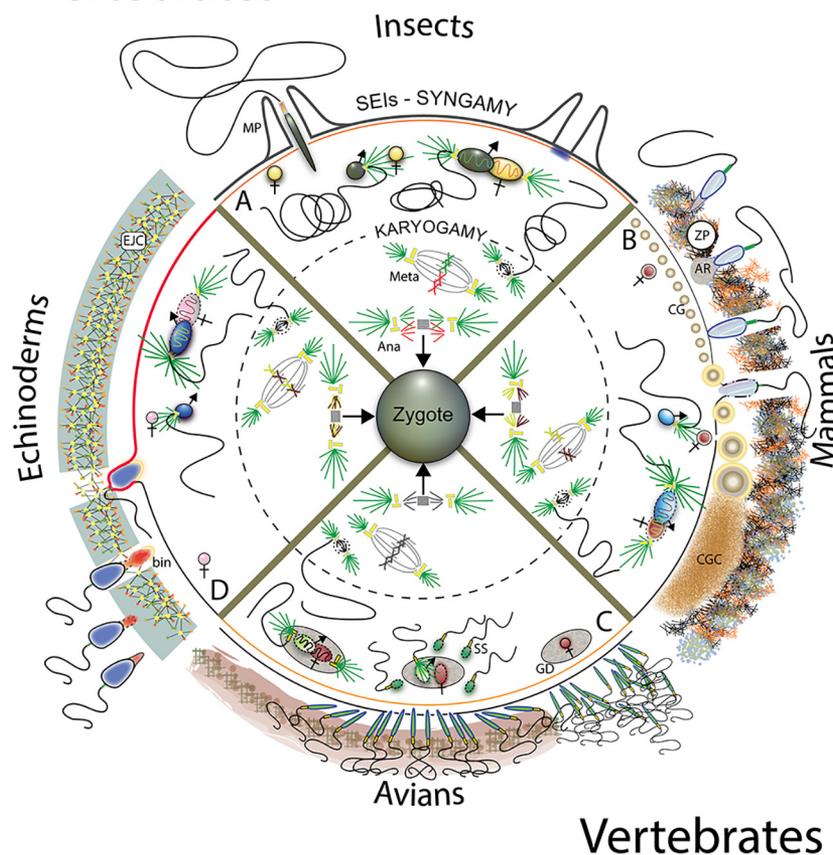
Whereas proteomics has become a mature scientific discipline in many fields (a recent PubMed search for keyword "proteomics" returned 80,000+ citations), addition of the keyword "sperm" returned only ~700 citations (<1.0%). Similar results were obtained using keywords "sexual reproduction and proteomics." Thus, as the title of this introduction implies, reproductive proteomics is truly "coming of age" but is clearly not yet there. Additionally, this issue is intended to illustrate the many practical applications and utility of proteomic technologies applied to human reproduction as both human *fertility* and *infertility* pose significant global health problems from two opposing directions—first, fertility in developing countries is the biological engine that drives population explosions thus producing associated societal and public policy issues impacting the human condition (7). Second, human infertility negatively impacts both individuals who desire children and globally, where some countries are struggling with population-wide reductions in birthrates as often reported in the popular press and the subject of intense study by demographers, statisticians, and sociologists (8). Proteomic approaches are particularly well-suited for the study of sexual reproduction because most interactions and interesting biology takes place almost exclusively at the protein-protein interaction level in luminal microenvironments without gene expression and genetic regulatory elements playing a major

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# Invertebrates



# Vertebrates

FIG. 1. **Sperm, egg, fertilization, and zygote formation across the animal kingdom.** Four major animal groups illustrate the wide variety of interactions and structures involved (see (6) legend for detailed description).

role once sperm are produced. Therefore, study of the male ejaculate, which includes both seminal fluid proteins (SFPs)<sup>1</sup> and sperm, are ideal subjects for proteomic analysis. The same is true for male-female interactions between the male ejaculate and the female reproductive tract where interactions again take place outside of the body in the luminal microenvironments found along the female reproductive tract. Proteomics is revolutionizing the depth of our understanding of reproductive processes in these two areas.

Given the enormous and challenging depth and complexity of the subject matter, not surprisingly the topics covered in this issue are equally divergent and include: (1) SFPs and post-testicular modifications, (2) sperm and spermatogenesis, and (3) egg activation, amniotic fluid, and the ovary. Studies of SFPs, primarily in insects, are featured in our first four contributions. Beginning with the pioneering works in *D. melanogaster*, (reviewed in (9, 10)) the genetic and molecular basis of specific SFPs has provided a wealth of knowledge about the function of these important molecules. Although *D. melanogaster*, because of the rich genetic heritage available,

was an obvious choice for these early functional studies of SFP action, recent advances in “omics” technologies and mass spectrometry have opened up entirely new possibilities for molecular female genetic studies in related organisms.

The contribution from Degner *et al.* (11) reports on the seminal fluid and sperm proteomes of the yellow fever mosquito, *Aedes aegypti*. A threat to human populations worldwide, *A. aegypti* transmits not only yellow fever virus, but also carries other viruses with similar negative impacts on human health including Dengue, Zika, Chikungunya, and West Nile viruses (12). Understandably, enormous efforts and resources have been put in place to eradicate these diseases with the focus primarily on eradication of the main vector, *A. aegypti* using a variety of biocontrol strategies. A major strategy is to interrupt or otherwise disable sexual reproduction of the vector and the authors rightly point out that a more intimate understanding of the molecular and cellular mechanisms of sexual reproduction could greatly accelerate these strategies. Using both transcriptomic and proteomic profiling of the male accessory gland and seminal fluids (including sperm) after transfer into females, the authors identified as many as 280 seminal fluid proteins, a significant increase in our knowledge base of this important class of proteins in mosquitos. A fol-

<sup>1</sup> The abbreviations used are: SFP, seminal fluid proteins; GPCR, G-protein coupled receptor, ECM, extracellular matrix.

lowing study by Karr *et al.* (13) identified >3000 high-confidence proteins from the *Drosophila pseudoobscura* male accessory gland. Bioinformatic and gene ontology was used to identify from the 163 putative SFPs, 32% of which overlapped with previously identified *D. melanogaster* SFPs, thus yielding a set of >100 putative novel SFPs identified by this approach. The authors also demonstrated that SFPs evolve more rapidly than other proteins produced by or contained within the accessory gland.

Entire ecosystems depend on the health and vitality of the group of hymenoptera that include bees, wasps and ants. Bees are estimated to pollinate as much as 75% of all agricultural plants (14) and the recent decline in bee populations because of the epidemic of colony collapse (15) has the potential to negatively impact human food chains and ecosystem stability. Polyandrous (*i.e.* female matings with multiple males) queens mate soon after hatching and therefore store sperm from multiple males. Therefore, each male contributes its own suite of SFPs and sperm, raising the possibility that sperm competition and sexual conflict could compromise the viability of stored sperm. Because the queens cannot subsequently re-mate later, they must maintain viable sperm over their lengthy lifetimes (many decades in some species) in order to optimize their reproductive output. How the queen mitigates the impact of sperm competition and manages sperm storage and use over such a long time period remains a mystery, although one way might be to selectively inactivate those SFP components involved in sperm competition. An excellent model system for the study of sperm storage and use is the hymenopteran ant, *Atta colombica*, used by Doselli *et al.* (16) in artificial insemination experiments and mass spectrometry analysis of male SFPs transferred to the female. Transferred proteins were then extracted and analyzed for abundance changes. They found a surprisingly small number of SFPs were targeted for degradation including two proteolytic serine proteases, a *SERPIN* inhibitor, and a semen-liquefying acid phosphatase. Their results suggest that these proteins might play key roles in mediating sexual conflict, thus enhancing sperm preservation during storage. Identification of SFPs in insects is complicated by their small size and often rapid processing both during and following insemination. SFPs also have an unknown dynamic range of action where some (*e.g.* enzymes) may exert their biological effects at very low concentrations whereas others (*e.g.* structural) may be required at elevated levels. Therefore, approaches with enhanced sensitivity for detection and quantitation would be an important step in the identification of SFPs. With this in mind, Sepil *et al.* (17) employed a novel approach using label-free quantitation of *D. melanogaster* male reproductive tissues both before and after mating. In addition to the previously reported SFPs, nine novel candidate SFPs of high-confidence and 42 additional putative candidates were identified in this study.

A unifying theme in mammalian reproduction is the essential process of sperm capacitation that occurs in the female reproductive tract following insemination. Capacitation, and therefore the ability for efficient fertilization, is dependent on post-testicular modification of mammalian sperm in the epididymis, a process that can result in hundreds of protein changes in the sperm proteome before and after transit through the epididymis (18). It has been known for more than two decades that capacitation also results in dramatic alterations in the sperm phosphoproteome ((19) reviewed in (20)). Although presumed restricted to mammalian lineages, recent work in reptiles raised the possibility that similar physiological changes occur during sperm activation in reptiles (21). In this issue, solid evidence is presented by Nixon *et al.* (22) that sperm from the Australian saltwater crocodile (*Crocodylus porosus*) undergo capacitation. First, the authors identified >1000 proteins in crocodile sperm and further identified alterations in the sperm phosphoproteome, many being like those previously identified in mammals. The expansion of the process of sperm capacitation beyond mammals by this study raises important questions about the evolutionary origins of sperm capacitation across the animal kingdom.

Proteomics applied to the study of human infertility is becoming increasingly prevalent and, in this issue, Barrachina *et al.* (23) quantified seminal fluid proteomes from fertile and infertile men using tandem mass-tagged LC-MS/MS. This data was used in a standard statistical approach to quantify and compare relative protein levels between fertile and infertile patients. The power of the standard approach is that it suffers from the “peptide-to-protein” inference algorithms that assume all peptides are in the intact protein. Given the known high levels of proteases in semen this problem is magnified, making direct comparisons problematic. To further assess this issue, the authors employed a novel strategy that identified stable-protein pairs using shared peptides between proteins and between samples to estimate the levels of heterogeneity existing in the seminal plasma proteome. Compared with normal semen, infertile semen samples had dramatically decreased levels of stable-protein pairs. This novel approach has the promise of a more personalized analysis of sperm dysfunction but will depend on future studies that can provide additional statistical confirmation.

Nixon *et al.*, (24) provide a detailed analysis of mouse epididymosomes, small vesicles secreted by the epididymis. They identified a total of 1640 epididymosome proteins and reported interesting pattern differences in the epididymosome proteome at various positions along the epididymis. Almost 150 proteins had significant differential abundance between caput and corpus epididymosomes, and 344 with differential abundance between corpus and cauda epididymosomes. As noted by the authors, they were also able to show a high concordance in proteome composition with a previous study of changes in the sperm proteome during epididymal transit (18). Taken together these results provide an improved and

larger proteome data set of known sperm proteins derived from the epididymis. Behavioral factors influencing human fertility are poorly defined. Here Shen *et al.* (25) provide an interesting analysis of the effect that short-term male abstinence has on the semen proteome and correlated this data with pregnancy outcomes following *in vitro* fertilization. They found that short-term abstinence of a few hours compared with longer periods of a few days resulted in improved sperm parameters including motile sperm count and sperm vitality among others. Quantitation of sperm proteomes revealed >300 differentially abundant proteins with the majority up-regulated. Although the cellular mechanisms activated by abstinence responsible for these observed differences are unknown, this new database promises to provide new targets for additional study of this important area of human behavior and reproduction.

One way sperm sense their environment and respond accordingly is through signal-transduction pathways. Urizar-Arenaza *et al.* (26) studied a class of metabotropic receptors, G-protein coupled receptors (GPCRs), a large class of biomolecules that respond to external stimuli and transduce signals across membranes. GPCRs are well known in a broad variety of biological processes although their specific roles in sperm physiology and function are not well studied. The authors chose to study the kappa-opioid receptor (KOR) using a specific agonist, the drug U50488H. They employed TMT labeling and LC-MS/MS for quantitation and titanium dioxide for phosphoprotein enrichment. Among the many intriguing changes found in the phosphoproteome in response to U50488H treatment, numerous sites affected were on proteins of known biological function including structural elements of sperm such as AKAPs and outer dense fiber proteins, phosphoglycerate kinases and regulatory subunits of the proteasome. Spermatogenesis in the testis involves extraordinary and highly regulated cell differentiation processes. Abnormal or unregulated disruption to the processes would be expected to result in sperm with impaired function. Regulation of such complex developmental pathways is controlled in part by phosphorylation and dephosphorylation events carried out by kinases and phosphatases, respectively. To better understand these processes Castillo *et al.* (27) profiled the phosphoproteome of adult human testes through all stages of spermatogenesis. This resulted in an impressive atlas of over 8000 phosphopeptides that mapped to >2500 phosphoproteins. This study also identified 174 phosphorylated kinases of which the cyclin-dependent kinase 12 (CDK12) and p21-activated kinase 4 (PAK4) were further studied. This study clearly defines a large and important landscape of phosphoregulation during sperm differentiation and provides potential targets for functional studies.

The last three contributions provide a welcome balance to the male-centricity apparent in the previous contributions (the cover art and overleaf provides an historical perspective on this subject). Nonetheless, clearly understanding female re-

production is as important, if not more, than study of male reproductive biology. Without question, only by combining the “details” from both sides of this complex fertility coin will true integration and understanding follow. Fortunately, these final articles show just how much promise and potential proteomics brings to the female side of the biological table and hopefully serve to inspire and act as a springboard for future studies.

How do sperm-egg interactions during fertilization, syngamy, and karygamy serve to activate the egg and begin the developmental process of the newly formed diploid zygote? Almost universally across the animal kingdom, fertilization results in a rise in  $Ca^{2+}$  levels that initiates the complex series physiological changes that follow. Although insect egg activation is not triggered by fertilization but instead by passage through the female reproductive tract, both trigger a rise in  $Ca^{2+}$  levels in the egg that sets off a series of downstream events mediated by various cascades of phosphatases and kinases. Here, Zhang *et al.*, (28) focus on a specific serine/threonine phosphatase, calcineurin encoded by the *canB2* gene in *D. melanogaster*. Although calcineurin is required for egg activation, precise knowledge of the molecular details is lacking. To further our understanding of these complex events, they compared CanB2 RNAi knockdown and wild-type eggs using global proteomic profiling and phosphopeptide enrichment. Their data reveals that calcineurin regulates, either directly or indirectly, hundreds of phosphosites during activation and identified among these were proteins involved in the regulation of egg activation, meiosis, and protein translation. These results show that calcineurin is a central player in the initiation of egg activation and remodeling of the proteome during these crucial early steps of zygotic life.

Given the World Health Organization’s ranking of female infertility as the fifth highest global disability, a deeper understanding of basic human ovarian biology and physiology is clearly indicated and served as motivation for the study Ouni *et al.* (29). Although essentially descriptive in nature, the >1500 proteins identified in this study represent the first in-depth proteomic database of the human ovary. This study also provided a description of the extracellular matrix (ECM) of the ovary, an important contribution because of the ECMs central role in follicle function (reviewed in (30)). Finally, although small samples sizes hindered statistical analyses, this study demonstrated an important correlation between frozen and fresh ovarian tissues with an approximate 70% overlap between the two proteomes raising the possibility for additional extended studies using frozen samples.

SFPs are not the only essential cellular secretions central to reproductive success. In oviparous amniotes, *i.e.* monotremes, birds, and reptiles, eggs must be bathed in a multipurpose protective amniotic fluid. The general functions of amniotic fluids are well known, *e.g.* protective, nutritive, and immune functions, but the overall protein composition is complex and poorly understood. Da Silva *et al.* (31) use LC-MS/MS to identify doz-

ens of proteins present in the chicken amniotic fluid proteome before the influx of massive amounts of egg white proteins. Importantly, they found that 48 of these were common to both chicken and humans amniotic fluids defining a target set of high-quality proteins with potentially conserved function in developing egg and fetus. One practical application of these proteins is to serve as biomarkers useful for monitoring the health and vitality of egg and developing embryo.

A final look at this issue's remarkable cover reminds us once again how the magic and mystery of sex—including the maddening complexity inherent in the “details”—continues to inspire both thought and creativity. The contributions in this issue were intended to add fuel and inspiration to the enterprise. Along with the increasingly rapid advances in the tools available for proteomic analyses, advancements in the molecular basis of sexual reproduction continue with a goal of eventually putting a small “d” in Diable.

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